nature cell biology

ATR and ATM regulate the timing of DNA replication origin firing

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Timing of DNA replication initiation is dependent on S-phase-promoting kinase (SPK) activity at discrete origins and the simultaneous function of many replicons[1,2]. DNA damage prevents origin firing through the ATM- and ATR-dependent inhibition of Cdk2 and Cdc7 SPKs[3,4]. Here, we establish that modulation of ATM- and ATR-signalling pathways controls origin firing in the absence of DNA damage. Inhibition of ATM and ATR with caffeine or specific neutralizing antibodies, or upregulation of Cdk2 or Cdc7, promoted rapid and synchronous origin firing; conversely, inhibition of Cdc25A slowed DNA replication. Cdk2 was in equilibrium between active and inactive states, and the concentration of replication protein A (RPA)-bound single-stranded DNA (ssDNA) correlated with Chk1 activation and inhibition of origin firing. Furthermore, ATM was transiently activated during ongoing replication. We propose that ATR and ATM regulate SPK activity through a feedback mechanism originating at active replicons. Our observations establish that ATM- and ATR-signalling pathways operate during an unperturbed cell cycle to regulate initiation and progression of DNA synthesis, and are therefore poised to halt replication in the presence of DNA damage.

Initiation of DNA replication in egg extracts from *Xenopus laevis* occurs asynchronously and with irregular spacing (approximately one origin per 10 kilobases (kb)[1,5]). Timing of origin firing is dynamically altered during S-phase, ensuring complete duplication of the genome[5,6]. The rate of replication in *Xenopus* egg extracts decreases with increasing concentration of nuclei templates, with no effect on the elongation rate. This suggests that one or more factors are limiting for origin firing[7]. We reasoned that these limiting factors might be active S-phase kinases (SPKs). The SPKs Cdk2 and Cdc7 are regulated by the Ataxia telangiectasia mutated (ATM)- and ATM and Rad3-related (ATR)-kinase pathways, in response to various forms of DNA damage[3,8]. Therefore, we treated unstressed extracts with caffeine (an inhibitor of ATM and ATR kinases) and measured the rate of nucleotide incorporation at 2,000 and 10,000 nuclei μl^{-1} (Fig. 1a). Caffeine increased the replication rate, especially with higher concentrations of template, suggesting that caffeine overcame the limiting factors; furthermore, it suggested that those factors might be downstream targets of ATM and ATR. Nuclei were smaller at a higher template concentration, but were not affected by caffeine (Fig. 1a, right).

Nucleoplasmic extract (NPE) is a concentrated nuclear extract that promotes initiation and replication on templates assembled in membrane-free egg cytosol[9]. Unwinding of a plasmid template in NPE indicates origin firing and can be visualized (in the presence of the DNA polymerase inhibitor aphidicolin) as highly supercoiled DNA[10]. Unwinding was monitored at different concentrations of the NPE (relative to template), with or without caffeine, and was more efficient when the ratio of NPE to assembled plasmid was higher (Fig. 1b). Addition of caffeine mimicked the increase in NPE concentration, promoting more origin firing (Fig. 1b). Furthermore, caffeine increased the rate of unwinding in NPE (Fig. 1c). These observations demonstrate that NPE contains rate-limiting factors for origin firing that can be overcome with caffeine.

To further demonstrate the increase in origin density with caffeine, the loading of Cdc45 onto chromatin was analysed. Cdc45, which assembles in an SPK-dependent manner on chromatin at an origin, is an indicator of origin firing[11]. Chromatin-bound Cdc45 was more abundant after caffeine treatment (Fig. 1d), consistent with an increase in the density of activated origins on the template.

To ensure that the nuclei used for these experiments did not contain damaged DNA that would trigger a checkpoint, we demonstrated that the sperm had no detectable ssDNA or double-strand breaks (DSBs; Supplementary Information, Fig. S1). Furthermore, the caffeine effect was limited to origin-dependent DNA synthesis (Supplementary Information, Fig. S2).

We reasoned that if caffeine promotes more rapid origin firing, a higher density of replicated DNA of similar lengths (at a given time point) should be observed, representing a higher number of simultaneously active replicons. To test this, nuclei were incubated in extracts with the reversible DNA polymerase inhibitor Ara–dCTP, chased with dCTP, and incorporation measured over time on an alkaline gel. Caffeine increased the quantity of replicated product to similar levels to the untreated control, consistent with an increase in the number of origins fired synchronously (Fig. 1e). The average replicon size in *Xenopus* egg extracts is approximately 10 kb[12], whereas the size of the nascent strands is smaller than 10 kb (Fig. 1e). This suggests that there

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Published online: 27 June 2004, DOI: 10.1038/ncb1145

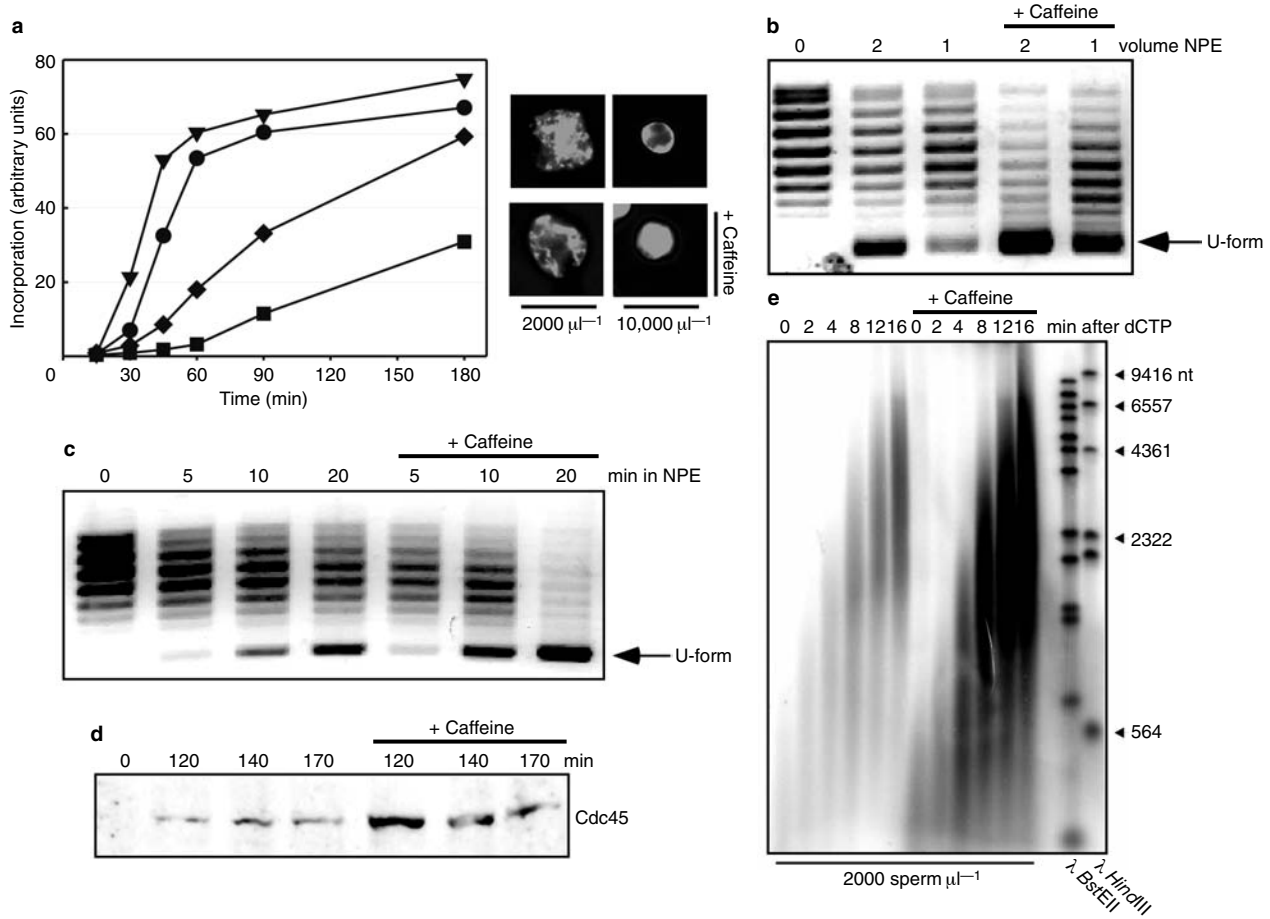

Figure 1 Caffeine accelerates the initiation of replication. (**a**) The rate of replication in *Xenopus laevis* egg extracts was measured by incorporation of α-^{32}P-dATP pulses at 0, 15, 30, 45, 60 and 90 min and stopped after 15, 30, 45, 60, 90 and 180 min, respectively. Products (20,000 nuclei/lane) were resolved on agarose gels, quantified using a Phosphorimager, normalized for nuclei, and the sum of the time points plotted (circle, 2,000 nuclei μl^{-1}; triangle, 2,000 nuclei μl^{-1} + 5 mM caffeine; square, 10,000 nuclei μl^{-1}; diamond, 10,000 nuclei μl^{-1} + 5 mM caffeine). Micrographs show assembled nuclei at 180 min after addition of 2,000 or 10,000 nuclei/μl in the absence or presence of 5 mM caffeine. (**b**) pBluescript-II plasmid was incubated in egg cytosol at 50 ng μl^{-1} before addition of single or twofold volumes of NPE containing aphidicolin with or without 5 mM caffeine. Reactions were stopped after 15 min and analysed as described[8] on a chloroquine–agarose gel (U-form is the unwound plasmid). (**c**) pBluescript-II was incubated in cytosol and one volume of NPE added with or without 5 mM caffeine. Aliquots of the reaction were removed at 5, 10 and 20 min and analysed on a chloroquine–agarose gel. (**d**) 10,000 nuclei μl^{-1} were incubated in egg extract, with or without 5 mM caffeine, and chromatin was isolated from replicating extracts at the indicated times and immunoblotted for *Xenopus* Cdc45 protein. (**e**) Analysis of nascent-strand synthesis on an alkaline gel was performed with 2,000 nuclei μl^{-1} in extract with α-^{32}P-dATP and 0.2 mM Ara–dCTP in the absence or presence of 5 mM caffeine. dCTP was added 40 min after addition of template and aliquots were taken at 2, 4, 8, 12 and 16 min.

is no notable fusion of replicons at these early times. Together, these results show that origin firing is rate-limited and that increased levels of nuclear factors, or the addition of caffeine, promotes faster initiation and replication of intact DNA templates.

The effect of caffeine suggested a function for ATM, ATR (or both) kinase pathways, in regulating origin firing. To test whether ATM and ATR kinases directly regulate normal origin-firing timing, components of their respective signalling pathways were modulated. Neutralizing antibodies that specifically target ATR[8] or ATM[13] (Supplementary Information, Fig. S3) to egg extracts induced a marked increase in nucleotide incorporation (Fig. 2a). These observations suggest that ATR and ATM proteins were at least partly responsible for the increased rate of replication promoted by caffeine.

We reasoned that ATR and ATM kinase activity must be regulated during DNA replication to explain their effect on incorporation. To monitor possible changes in activity, Histone H2AX phosphorylation was assessed in extracts during replication of undamaged sperm nuclei[14]. In untreated extracts, total H2AX kinase activity increased as replication proceeded (Fig. 2b). Mock-antibody treatment did not affect the activity, whereas addition of ATR-neutralizing antibodies substantially reduced the kinase activity during replication. Addition of ATM-neutralizing antibodies reduced the kinase activity only at later times. In the absence of added sperm nuclei, no kinase activity was observed above background levels (data not shown).

Furthermore, we demonstrated that in the absence of damage ATM became autophosphorylated and activated during replication. A replicating extract was treated with okadaic acid (a phosphatase inhibitor) at levels that inhibit both PP1 and PP2A enzymes, to trap transient auto-phosphorylation of ATM. In the absence of okadaic acid, no autophosphorylation of ATM was observed (Fig. 2c). After okadaic acid treatment, a modest phosphorylation of ATM on Ser 1,981 was observed that was reduced with caffeine (Fig. 2c). In comparison, in

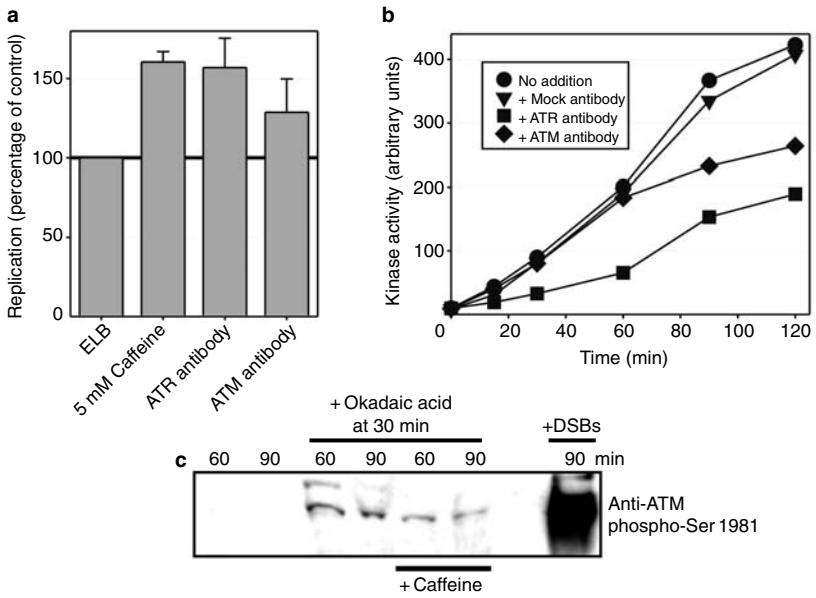

Figure 2 The effect of caffeine on replication is the result of ATM and ATR inhibition. (**a**) 10,000 nuclei μl^{-1} were incubated in extract with α-^{32}P-dATP for 1 h (in duplicate) with ELB buffer, 5 mM caffeine, 20% (v/v) ATR-neutralizing antibody, or 20% (v/v) ATM-neutralizing antibody. Replication products were quantified and plotted as a percentage of buffer control. (**b**) Interphase egg extracts were incubated with sperm nuclei at 10,000 μl^{-1}, in the absence (circle) or presence (diamond) of ATM-neutralizing antibody, ATR-neutralizing antibody (square), or a mock antibody (total non-immunized rabbit IgG; triangle). Kinase activity towards a peptide derived from Histone γ-H2AX was assayed at the times indicated during sperm replication. (**c**) Sperm nuclei were incubated in egg extract at 10,000 μl^{-1} with or without 5 mM caffeine. Okadaic acid (4 μM) was added to the indicated samples after 30 min and 1.5 μl was removed for analysis after 60 or 90 min. Double-strand-break-containing DNA (40 ng μl^{-1}) was added to egg extract and 1.5 μl removed for analysis after 90 min. These samples were probed for the presence Ser 1,981-phosphorylated ATM.

the absence of okadaic acid, a persistent phosphorylation of ATM Ser 1,981 was observed after addition of un-repairable DSBs (Fig. 2c), demonstrating the strong activation of ATM after DNA damage.

The ultimate downstream targets of the ATM and ATR pathways in regulating DNA replication include the Cdk2 and Cdc7 kinases. Addition of recombinant Cdk2–Cyclin-E and Cdc7–Dbf4 to the unwinding reaction increased the rate of initiation relative to the control (Fig. 3a). In addition, Cdk2–Cyclin-E promoted a dose-dependent increase in unwinding (Fig. 3b). Addition of Cdc45, which is loaded as a consequence of SPK action, had no affect on unwinding (Fig. 3a). Addition of Cdk2, Cdc7 and Cdc45 to the reaction promoted an even more rapid initiation, resulting in complete unwinding within 5 min (Fig. 3a). These data strongly support the hypothesis that Cdk2 and Cdc7 kinases are limiting factors in promoting origin firing.

Addition of recombinant Cdc25A — the phosphatase that removes the inhibitory phosphate from Tyr 15 of Cdk2 and is targeted for inhibition by the ATM and ATR pathways[3] — induced an increase in unwinding and replication similar to that promoted by caffeine (Fig. 3c and data not shown); conversely, addition of catalytically inactive Cdc25A inhibited unwinding (Fig. 3c). NSC663284, a potent chemical inhibitor of Cdc25A[15], markedly reduced replication. The specificity of the inhibitor was demonstrated by addition of recombinant Cdc25A, which restored incorporation (Fig. 3d). This observation suggests that Cdc25A activity is necessary for normal DNA replication. Comparing the kinetics of nucleotide incorporation between caffeine- and NSC663284-treated extracts showed that caffeine increased the rate of replication, whereas NSC663284 reduced the rate of replication (Fig. 3e), although the endpoint was the same in both situations. These observations are consistent with ATM and ATR directly regulating timing of replication in the absence of DNA damage, partly through downstream Cdc25A activity.

To demonstrate directly that these effects resulted from modulation of an SPK by the ATM and ATR pathways, Cdk2–Cyclin-E was isolated from NPE with p13^{suc1}–agarose beads[16] and kinase activity was measured. p13^{suc1} binds to active Cdk2–Cyclin-E and Cdc2–Cyclin-B complexes with high affinity. NPE prepared from cycloheximide-treated extracts lacks Cyclin B; therefore, only Cdk2–Cyclin E binds to p13^{suc1} beads. The inhibition of ATM and ATR with caffeine or neutralizing antibodies increased Cdk2 activity, whereas addition of NSC663284 decreased Cdk2 activity (Fig. 3f). This observation demonstrated that ATR affects the activity of Cdk2 directly. Finally, the inhibitory phosphorylation on Tyr 15 of Cdk2 was significantly reduced in nuclei isolated from caffeine-treated extracts, relative to those isolated from control extracts; furthermore, it was increased moderately in nuclei isolated from NSC663284-treated extracts (Fig. 3g). These data suggest that SPKs exist in an equilibrium between active and inactive states, and that ATM and ATR signalling pathways modulate these states.

ssDNA is a replication intermediate[10] found in complex with the ssDNA-binding protein RPA. ATR activity is stimulated by RPA-bound ssDNA[8,17] and RPA is transiently bound to chromatin during replication[8]. Therefore, we reasoned that this transient RPA–ssDNA structure may promote localized ATR activity and thus modulate the activity of SPKs on neighbouring origins. Therefore, we measured the quantity of ssDNA that accumulated transiently during replication by monitoring RPA accumulation on undamaged chromatin incubated in extract. Binding of RPA to chromatin peaked as replication initiated (Fig. 4a, top), consistent with a burst of initiation events and localized melting of DNA. Treatment of the replicating sperm with caffeine resulted in an

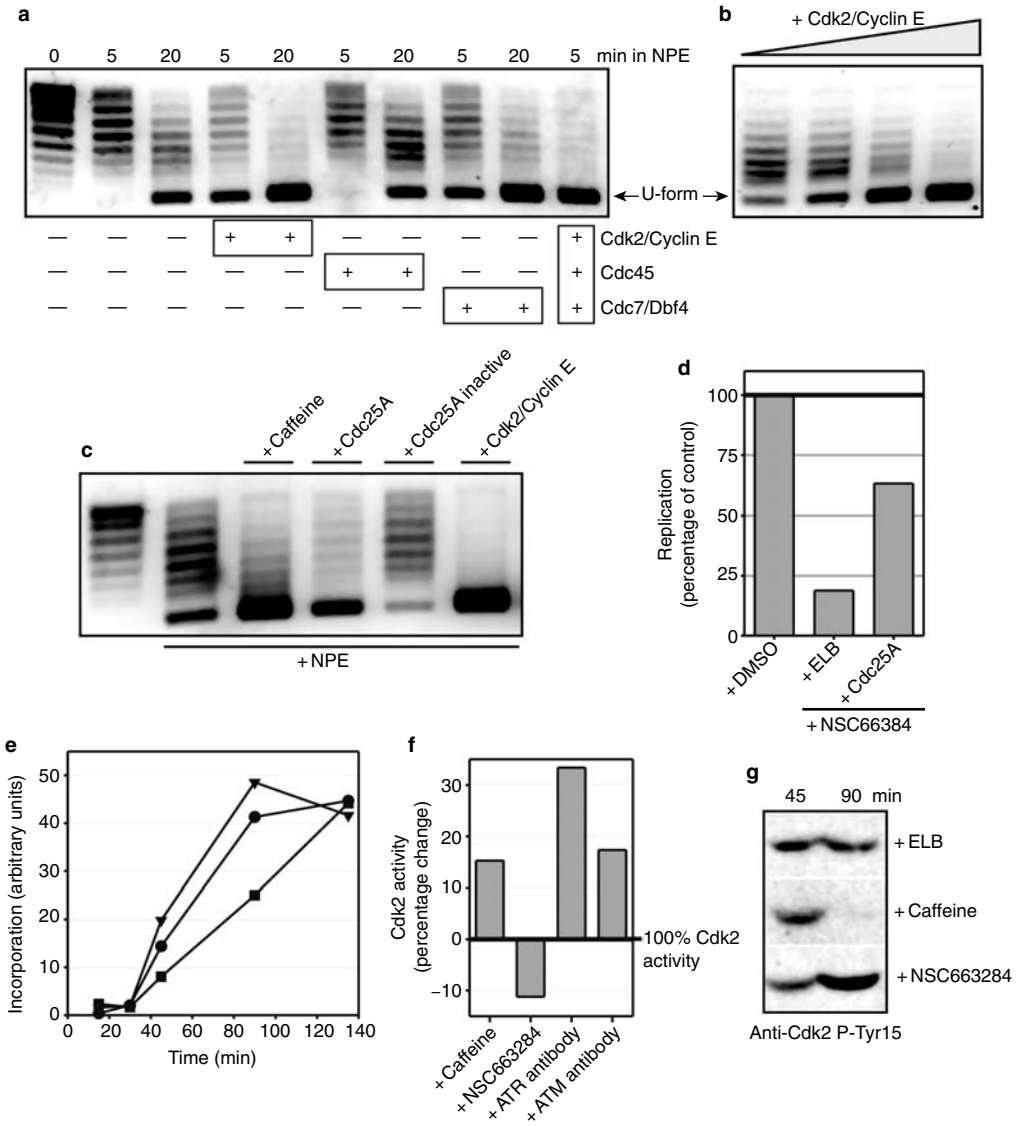

Figure 3 Regulation of firing and replication by rate-limiting SPKs and Cdc25A. (**a**) pBluescript-II was incubated in cytosol before two volumes of NPE were added, along with recombinant Cdk2–Cyclin-E (80 ng μl^{-1}), Cdc45 (50 ng μl^{-1}) or Cdc7–Dbf4 (20 ng μl^{-1}). Reactions were stopped after the indicated times and analysed on a chloroquine–agarose gel. (**b**) The unwinding reaction was performed after addition of recombinant Cdk2–Cyclin-E (1.1, 3.3, 10 or 30 ng μl^{-1}) to the NPE (lanes 1–4, respectively), and incubated for 20 min. (**c**) pBluescript-II was incubated in cytosol for 1 h before one volume of NPE was added with 5 mM caffeine, Cdc25A protein, catalytically inactive mutant Cdc25A protein[13], or Cdk2–Cyclin-E. Reactions were stopped after 20 min and analysed on a chloroquine–agarose gel. (**d**) 10,000 nuclei μl^{-1} were incubated in interphase extracts for 1 h with α-^{32}P-dATP, 0.19% DMSO in ELB, 60 μM NSC663284, or with 60 μM NSC663284 + 66 ng μl^{-1} Cdc25A. Reactions were stopped after 1 h and quantified as a percentage of the buffer control. (**e**) 2,000nuclei μl^{-1} were incubated in extracts containing α-^{32}P-dATP with ELB (circle), 5 mM caffeine (triangle), or 100 μM NSC663284 (square), as indicated. Aliquots were removed and stopped after 15, 30, 45, 90, and 135 min, and incorporation quantified and plotted. (**f**) NPE was incubated alone, with 5 mM caffeine, 100μM NSC663284, 20% (v/v) ATR-neutralizing antibody, or 20% (v/v) ATM-neutralizing antibody for 30 min. p13^{suc1}-agarose beads were then added to isolate Cdk2 (ref. 35). A kinase assay with isolated Cdk2 was quantified and plotted as a percentage change from the control. (**g**) Nuclei were isolated from extracts at 45 and 90 min after addition of 10,000 nuclei μl^{-1} with ELB, 5 mM caffeine or 100 μM NSC663284, and immunoblotted for Tyr-15-phosphorylated Cdk2.

increase in RPA binding (Fig. 4a, top), consistent with greater amounts of simultaneously active replicons. Etoposide — which blocks DNA Topoisomerase II, resulting in ssDNA stretches and ATR-checkpoint activation — was added as a positive control for RPA binding and checkpoint activation[8] (Fig. 4a, top). In parallel, activity of Chk1 kinase was measured in the same replicating extract. Chk1 is a downstream effector kinase of the ATR pathway, and is activated by phosphorylation on Ser 344 (the equivalent residue to human Ser 345)[18]. Activation of Chk1 correlated with the timing of RPA accumulation, whereas caffeine treatment mostly eliminated Chk1 activation (Fig. 4a, bottom), consistent with transient ssDNA being the signal that downregulates origin firing. Caffeine also inhibited the activation of Chk1 after the addition of etoposide (Fig. 4a, far-right-hand lane).

To further demonstrate that ssDNA slows DNA replication, M13 ssDNA was added to egg extract. An inhibition of chromosomal DNA replication that could be rescued with caffeine was observed (Fig. 4b). This suggests that ssDNA signals *in trans* through a caffeine-sensitive pathway to downregulate replication. As M13 ssDNA might function

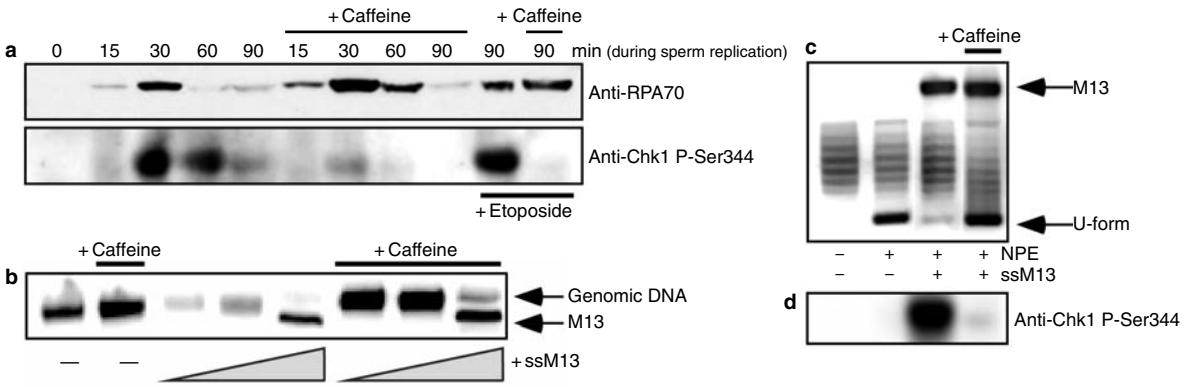

Figure 4 ssDNA transiently accumulates and modulates origin firing. (**a**) 10,000 nuclei μl^{-1} were incubated in interphase egg extracts with or without 5 mM caffeine, or with 30 μM etoposide, as indicated. Chromatin was isolated from 40 μl of the reaction at the indicated times and immunoblotted for the presence of Rpa70. An aliquot (1 μl) of the total reaction was immunoblotted for the presence of Ser-344-phosphorylated Chk1. (**b**) 2,000 nuclei/μl were incubated in extracts with α-^{32}P-ATP and 25, 2.5 or 0.25 ng μl^{-1} M13 ssDNA with or without 5 mM caffeine. The reaction was stopped after 1 h and products were resolved on an agarose gel. Replicated M13 DNA migrated ahead of the sperm DNA. (**c**) pBluescript-II was incubated in cytosol before two volumes of NPE (pre-incubated with 25 ng μl^{-1} M13 ssDNA) were added with or without 5 mM caffeine. The reaction was stopped after 15 min and analysed on a chloroquine–agarose gel. (**d**) A reaction was performed as in **c** and 0.5 μl aliquots were immunoblotted for Ser-344-phosphorylated Chk1.

to titrate necessary replication factors, we also probed the effect of M13 ssDNA addition on an unwinding assay performed in the presence of the replication inhibitor aphidicolin. Addition of M13 ssDNA reduced the extent of unwinding, an effect that was rescued with caffeine (Fig. 4c). Addition of M13 ssDNA also induced a marked increase in the activation of Chk1, an effect that was reversed with caffeine (Fig. 4d). Together, these observations support the hypothesis that ssDNA is a trigger, mediated by the ATR-pathway, that downregulates origin firing.

ssDNA accumulates after treatment with the DNA polymerase inhibitor aphidicolin, probably owing to de-coupling of the helicase from the polymerase[10]. High levels of aphidicolin (100 ng μl^{-1}) that completely block synthesis induce a checkpoint[19,20] triggered by the accumulated ssDNA[8,17]. We reasoned that transient ssDNA generated during replication might modulate SPK activity during replication. Therefore, we tested the effect of aphidicolin at levels that partially inhibit DNA polymerases and slow the rate of nucleotide incorporation (but do not halt it), resulting in increased levels of ssDNA (Fig. 5d, top). These levels of aphidicolin have been demonstrated not to induce a checkpoint. When extracts were treated with 10 ng μl^{-1} aphidicolin, the rate of nucleotide incorporation was limited but could be reversed with caffeine. Treatment with 1 ng μl^{-1} aphidicolin modestly reduced the rate of replication, but was still rescued with caffeine (Fig. 5a, b). In the absence of aphidicolin there was a minor increase in the rate of nucleotide incorporation after the addition of caffeine (Fig. 5a, bottom; also see Fig. 1a). Finally, at 100 ng μl^{-1} aphidicolin, replication was entirely inhibited and was not rescued with caffeine (Fig. 5c and data not shown). These data further suggest that inhibition of origin firing correlates with the extent of ssDNA accumulation.

To verify that the inhibition of initiation was coincident with appearance of ssDNA, chromatin was isolated, incubated in extract with increasing levels of aphidicolin (in the presence or absence of caffeine), immunoblotted for RPA, and incorporation measured simultaneously (Fig. 5c). The amount of chromatin-bound RPA increased with the levels of aphidicolin (Fig. 5d, top), consistent with aphidicolin causing runaway helicase action and an increased quantity of ssDNA. Addition of caffeine to the reaction modestly increased the amount of chromatin-bound RPA, consistent with an increased density of caffeine-induced activated origins (Figs 4a and 5d, top). Notably, analysis of total extracts from the same experiment identified a marked increase in activated Chk1, concomitant with the increased levels of ssDNA, which was abrogated after addition of caffeine (Fig. 5d, middle). Intriguingly, the inhibitory phosphate on Tyr 15 of Cdk2 was present at a constant level independently of aphidicolin, and this level was slightly reduced after treatment with caffeine (Fig. 5d, bottom). This suggests that Cdk2 modulation normally occurs during replication but is not responsive to ssDNA levels, whereas Chk1 activity is very responsive to ssDNA levels.

To further show that the effect of caffeine in promoting increased origin firing is partly the result of feedback from ssDNA–RPA intermediates, we depleted RPA from the extract. After partial depletion of RPA from the egg cytosol and from NPE (data not shown), appearance of the U-form of DNA was inhibited, whereas addition of recombinant human RPA (hRPA) rescued unwinding (Fig. 5e). Addition of caffeine to the depleted extracts did not promote increased appearance of the U-form, whereas addition of caffeine and recombinant hRPA did promote increased unwinding (Fig. 5e), consistent with the requirement for RPA-bound ssDNA in ATR activation[17].

These data support the hypothesis that ssDNA, a normal metabolic intermediate of DNA replication, is responsible for attenuating origin firing by stimulating ATR and modulating origin firing activity through Chk1 and its downstream targets.

Our results show that DNA damage checkpoint pathways that regulate S-phase progression are not just binary switches that either permit origin firing and cell-cycle progression, or completely block replication initiation after sensing damage. Indeed, ATR and Chk1 (a kinase in the ATR signalling pathway) are essential in mice[18,21]; this suggests further roles in addition to those in the response to exogenous DNA damage. In addition, ATR has been implicated in the regulation of slow-zone replication[22], and Chk1 inhibits Cdc25A activity in an unperturbed cell cycle[23,24].

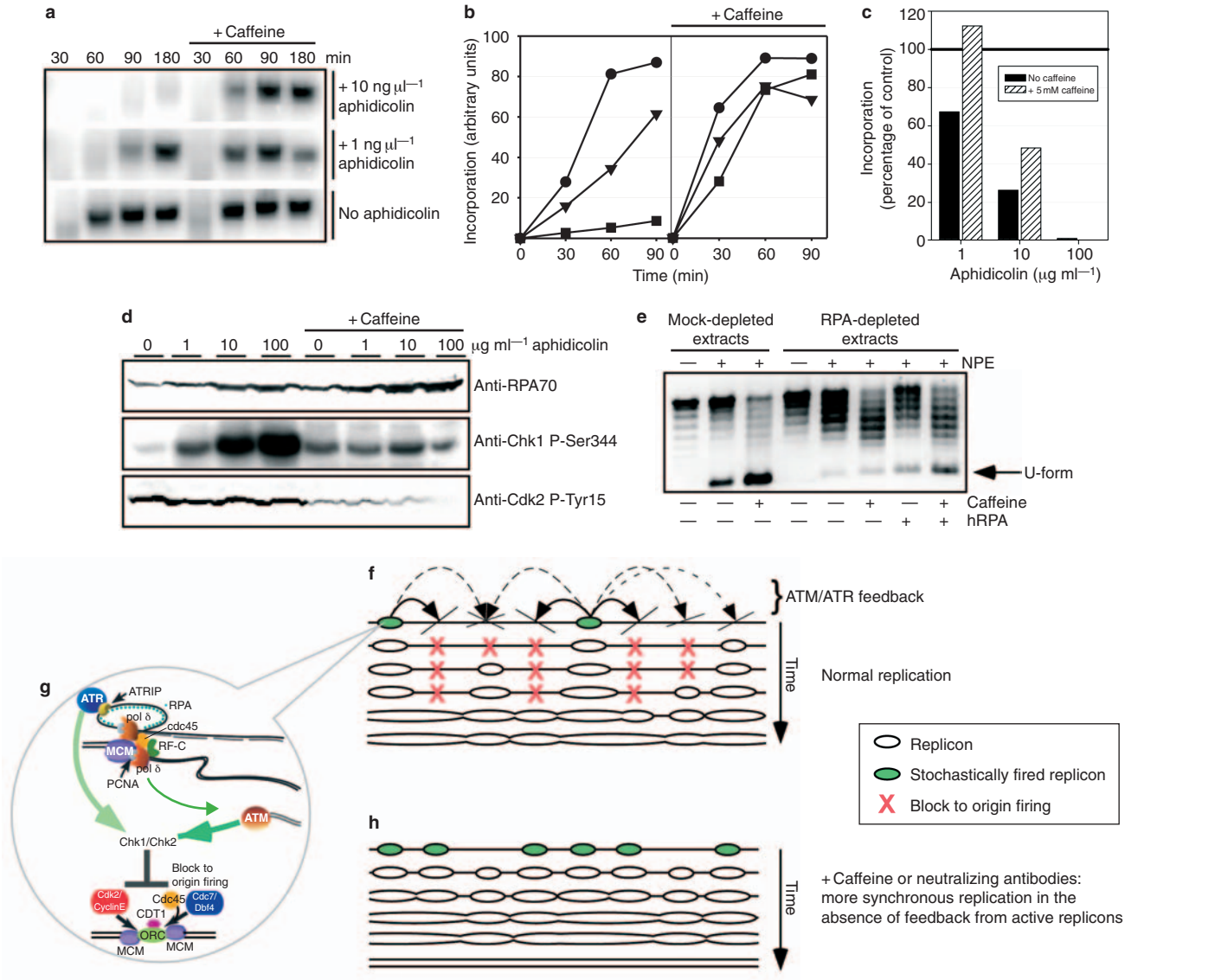

Figure 5 ssDNA regulates the caffeine-sensitive inhibition of origin firing. (**a**) 2,000 nuclei μl^{-1} were incubated in extract with α-^{32}P-ATP with or without 5 mM caffeine and with ELB, 1 ng μl^{-1} aphidicolin, or 10 ng μl^{-1} aphidicolin. Aliquots (5 μl) of each reaction were stopped after 30, 60, 90 or 180 min, and resolved on a 0.8% agarose gel. (**b**) The incorporation in each reaction lane was quantified and plotted. The graph illustrates the kinetic profile of incorporation, with varying levels of aphidicolin (circle, no aphidicolin; triangle, 1 ng μl^{-1} aphidicolin; square, 10 ng μl^{-1} aphidicolin), with or without caffeine. (**c**) 10,000 nuclei μl^{-1} were incubated in extract with α-^{32}P-dATP, with or without 5 mM caffeine, and with ELB and aphidicolin (1, 10 or 100 ng μl^{-1}). Reactions were stopped after 1 h, and analysed as in **b**, and incorporation plotted as a percentage of the control without aphidicolin. (**d**) Chromatin, from 500,000 total nuclei was isolated from the reaction in **c** and probed for an RPA subunit (relative molecular mass of 70,000) at various concentrations of aphidicolin. Aliquots of total extract (2μl) from the same reactions were immunoblotted for Ser-344-phosphorylated Chk1 and for Tyr-15-phosphorylated Cdk2. (**e**) pBluescript-II was incubated in mock-depleted or RPA-depleted cytosol before two volumes of mock-depleted or RPA-depleted NPE containing aphidicolin were added with or without caffeine, and with or without 125 ng of purified hRPA protein. (**f**) A model for the role of ATR and ATM in the dynamic regulation of origin firing: at entry into S-phase, some origins are stochastically fired after the action of the SPKs, and DNA replication proceeds. Feedback from active replicons results in the inhibition of origin firing on adjacent origins. (**g**) Transient ssDNA generated during DNA replication and occasional DSBs that arise (possibly after fork collapse) result in modest and localized ATR and ATM activation, respectively. We have previously discussed possible causes of DSB accumulation[27]. ATR and ATM pathway activities downregulate Cdk2 and Cdc7, and block their ability to activate unfired origins. (**h**) In the presence of caffeine, or after specific inhibition of ATR and ATM, the feedback inhibition of origin firing from active replicons is relieved and more origins can fire simultaneously.

We have demonstrated the following evidence in support of a function of ATM in regulating normal origin firing: first, specific inhibition of ATM with neutralizing antibodies significantly increased DNA replication; second, addition of active recombinant ATM protein rescued the positive effects of both caffeine and the ATM-neutralizing antibodies; third, ATM-associated kinase activity increased as DNA replication proceeded; and finally, ATM was transiently autophosphorylated during ongoing replication. Taken together, these observations support the hypothesis that ATM is normally, but transiently, active in an undamaged context.

This observation is surprising as *ATM*$^{-/-}$ cells do not have a major cell-cycle defect other than radio-resistant DNA synthesis[3]. However,

earlier studies do indicate a modest increase in DNA replication intermediates in non-irradiated cells derived from ataxia telangiectasia patients with mutant ATM protein[25,26]. In addition, we have previously shown that double-strand breaks normally arise during S-phase[27]. We now propose that the transient generation of DSBs during DNA replication triggers a local activation of ATM on replicating chromatin, which is consistent with the kinetics of chromatin-bound ATM activation.

Together, our data provides strong evidence that two distinct pathways regulate normal replication origin firing in concert. ATR, most probably activated by feedback from transiently generated ssDNA at previously initiated replicons, functions to slow the rate of replication by downstream inhibition of Cdc7 at later firing origins. Similarly, ATM slows the rate of replication by inhibiting the Cdk2 kinase (Fig. 5g).

Mini chromosome maintenance (Mcm) proteins accumulate at sites on the DNA that are more closely spaced than necessary for origins that are actually used. This suggests that origins are dynamically selected, possibly in accordance with the 'origin interference' model[28]. Our observations — that origin firing is regulated by ATR and ATM through feedback from ssDNA and other replication intermediates — fit with this model (Fig. 5f–h). Therefore, origins in *Xenopus* egg extracts may be zones of unwinding that promote RPA binding, concomitant with a feedback mechanism from this localized DNA melting to prevent further origin activation events nearby and ensuring a regular spacing of replicons.

In summary, we find that in the absence of DNA damage, the rate of replication and unwinding is regulated by the caffeine-sensitive ATM and ATR pathways. We propose that ATR and ATM regulate SPK activity through feedback from stochastically fired replicons. Our observations establish that the ATM and ATR pathways operate during an unperturbed cell cycle to regulate initiation of DNA replication.

Note added in proof: While this manuscript was under review, a study by Marheineke and Hyrien[29] reached similar conclusions.

METHODS

Antibodies and reagents. Anti-phospho-Tyr 15, anti-Cdk2 and anti-phospho-Ser-345 Chk1 (Ser 344 in *Xenopus* Chk1) antibodies were purchased from Upstate (Lake Placid, NY). Anti-RPA70 serum was a gift from P. Jackson, anti-Cdc45 serum was a gift from H. Takisawa and anti-phospho-Ser-1,981 ATM was from Rockland Immunochemicals (Gilbertsville, PA). Anti-ATR antibodies were prepared from a carboxy-terminal peptide and specifically neutralized ATR activity as described[8]. Anti-ATM antibodies were prepared from a region of ATM that is not homologous to ATR[30], and specifically neutralized ATM activity as described[13]. Mock antibodies were prepared as total IgG purified from non-immunized rabbits.

Caffeine was purchased from Sigma (C-0750; St Louis, MO) and dissolved in 10 mM PIPES at pH 8.0; α-^{32}P-ATP was purchased from Amersham Biosciences (Piscataway, NJ); SYBR–Gold was purchased from Molecular Probes (Eugene, OR); Ara–dCTP (cytosine β-D-arabinofuranoside 5′-triphosphate) was purchased from Sigma (C-3639); M13 single-stranded DNA was from New England Biosciences, NSC663284 (6-chloro-7-(2-morpholin-4-ethylamino)-quinoline-5,8-dione) was provided by the Drug Synthesis & Chemistry Branch of the National Cancer Institute and dissolved in 100% dimethyl sulphoxide at a stock concentration of 30 mM. NSC663284 was further diluted into ELB buffer as required. Okadaic acid, as a sodium salt (O-7760; Sigma), was dissolved in water at a stock concentration of 100 μM.

Extract and nuclei preparations. All extract incubations were performed at 22 °C. Interphase *Xenopus* egg extracts were prepared as described[31] in the presence of cycloheximide. Before use, each batch of extract was assayed for competent and timely nuclear assembly, and only extracts that formed rounded nuclei with a nuclear envelope in 30 min were used. Membrane-free egg cytosol and NPE were prepared as described[9,31]. Sperm nuclei were prepared as described[32], except that de-membranation was performed with 0.2% Triton X-100 for 15 min. Exonuclease-III-treated nuclei were prepared as described[8]. For isolation of nuclei from extracts, sperm were incubated in interphase extracts for various times and conditions, as indicated, and 40 μl were immediately diluted in up to 1 ml of ice-cold egg lysis buffer (ELB; 200 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes at pH 7.8, 1 mM dithiothreitol (DTT) and 50 mg μl^{-1} cycloheximide) and under-layered with ELB containing 500 mM sucrose. Nuclei were pelleted at 8,000*g* for 5 min and washed once in 1 ml ELB before re-pelleting. The final pellet was resuspended in SDS–PAGE loading buffer, resolved on a 10% SDS–PAGE gel and analysed by immunoblotting with specific polyclonal antibodies, as indicated.

Extract assays. For RPA-depletion of egg extracts, three volumes of RPA serum were pre-incubated with protein A–Sepharose and beads washed with ELB. Egg cytosol and NPE were each subjected to two rounds of incubation with one volume of beads bound to RPA antibody.

For replication assays in interphase extracts, the extract was supplemented with energy mix[31], de-membranated sperm nuclei (to the required concentration) and α-^{32}P-ATP. Reactions were incubated at 22 °C, and 20,000 nuclei were stopped in 200 μl 1% SDS, 40 mM EDTA and 10 mM Tris-HCl at pH 8.0. The reaction was analysed as described[8].

Ethanol-precipitated replication products were resolved on a 0.7% alkaline agarose gel. Lambda DNA markers, digested with *BstE*II or *Hind*III, were end-labelled with T4 Kinase.

Density substitution. Replication was performed in interphase extracts or NPE and deproteinized as described above. One third of the reaction was mixed with caesium chloride dissolved in Tris-HCl at pH 8.0, 0.5 mM EDTA at 1.72g cm^{-3}, loaded into a 3.5-ml quick-seal ultracentrifuge tube (Beckman, Fullerton, CA) and centrifuged for 48 h at 82,000*g*. Fractions (each typically 175 μl) were removed from sequentially from the top of the tube and analysed as described[9].

Chromatin binding assays. The chromatin binding assay for Cdc45 was performed as described[13]. The chromatin binding assay for RPA was performed by diluting 40 μl of extract containing 10,000 nuclei μl^{-1} into 800 μl of ice-cold ELB containing 0.125% Triton X-100, 1 mM spermine and 1 mM spermidine, and incubated on ice for 5 min. This was under-layered with 200 μl of ELB containing 0.5 M sucrose and centrifuged at 10,500*g* for 5 min. The supernatant was removed then the isolated chromatin was analysed by immunoblotting with the indicated antibodies.

Plasmid replication and unwinding assays in NPE. For replication[9] and unwinding assays[10], plasmid DNA (pBluescriptII, all migrating as supercoiled DNA on an agarose gel) was purified using a Qiagen kit (Qiagen, Valencia, CA) and assays performed as described. NPE contained 50 ng μl^{-1} aphidicolin in all unwinding assays.

ATM and ATR phosphorylation assays. All kinase assays and peptide phosphorylation assays were performed at 30 °C, and DSBs were generated by blunt-end restriction digestion of plasmid DNA, as described[13]. For the kinase assays, 2 μl of treated extract was mixed with 20 μl of EB kinase buffer (EB Kinase buffer contained 20 mM Hepes at pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4 and 10 mM $MnCl_2$) supplemented with 0.5 mg ml^{-1} Histone H2A.X-derived peptide (C-terminal amino acids: AVGKKASQASQEY; Sigma), 50 μM ATP and 1 μl of α-^{32}P-ATP (10 mCi ml^{-1}, >3,000 mCi mM^{-1}). Samples were incubated at 30 °C for 20 min and assays were stopped (by addition of 20 μl of 50% acetic acid) and spotted onto P81 phosphocellulose filter paper (Upstate). Filters were air-dried, washed three times in 10% acetic acid and incorporation was quantified in a scintillation counter.

All radionucleotide-containing gels were dried and analysed in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Image intensities were adjusted in Adobe Photoshop and levels of incorporation quantified with ImageQuant software (Molecular Dynamics).

Protein for analysis of ATM phosphorylation was resolved on a 3–8% Tris–acetate NuPAGE gel according to the manufacturers instructions (Invitrogen, Carlsbad, CA). Immunoblots for phospho-Ser 344 Chk1 and phospho-Ser 1,981 ATM were performed with ECL Advance reagent (Amersham, Piscataway, NJ).

Production of recombinant proteins. Recombinant Cdk2–Cyclin-E was prepared as described[13]. The recombinant protein did not contain any Tyr 15 phosphorylation that was detectable by western blot. Recombinant Cdc7–Dbf4 was prepared as described[8]. *Xenopus laevis* Geminin was expressed in BL21(DE3) cells from a pET28A plasmid (a gift from M. Michael), according to standard protocols. Hexahistidine-tagged human $p21^{cip1}$ protein was expressed in BL21(DE3) cells, solubilized from inclusion bodies in 8 M urea, purified on a nickel column, renatured in aqueous solution using non-detergent Sulfobetaine (NDSB201, Calbiochem) as described[33] and concentrated on a MonoQ column (Amersham). Recombinant human ATM protein, a generous gift from T. Paull, was prepared exactly as described[34]. Recombinant human RPA protein was a generous gift from J. Boroweic.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank J. Walter for help and advice in setting up the NPE system; G.-H. Lee and T.T. Paull for the generous gift of purified ATM protein; K. Cimprich for the ATR antibody; J. Maller for Cdc25A baculovirus; M. Michael for the Geminin construct; D. Grieco for the p21 construct; H. Takisawa for the Cdc45 antibody; P. Jackson for RPA antibody; J. Boroweic for purified hRPA protein; E. Kim for production of recombinant Cdk2–Cyclin E and Cdc25A; and C. Ying for recombinant Cdc45. We also thank members of the Gautier lab for helpful discussions and critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (RO1 CA92245) and the American Cancer Society (RSG CCG-103367) to J.G.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial ineterests.

Received 5 May 2004; accepted 1 June 2004
Published online at http://www.nature.com/naturecellbiology.

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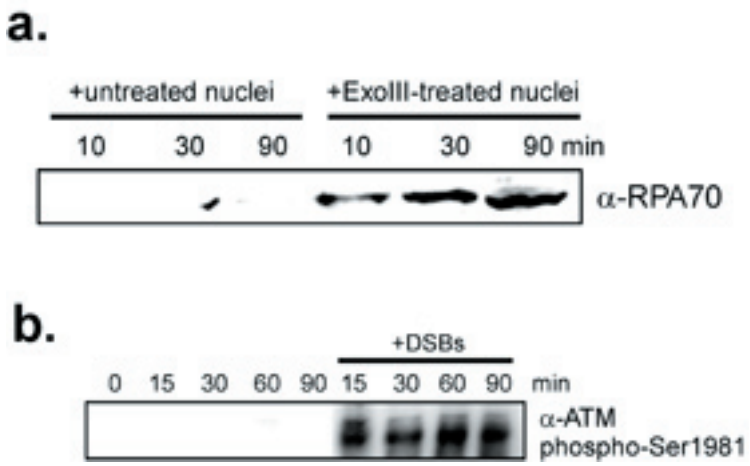

Figure S1 The chromatin template does not contain detectable ssDNA and does not trigger ATM activation upon incubation in extract. **(A)** Demembranated sperm nuclei or exonuclease-III treated sperm nuclei were incubated with membrane-free egg cytosol at 10,000/μl. The chromatin was isolated at 10, 30, and 90 minutes and probed for the presence of the RPA 70 kDa subunit. This shows that chromatin assembled in membrane-free egg cytosol, which does not support DNA replication, did not bind RPA at early time points. In contrast, exonuclease-III treated nuclei (with ssDNA gaps that activate an ATR-dependent checkpoint[1]) did promote rapid RPA binding, with a strong signal after 15 minutes. We conclude that there was no residual ssDNA in the nuclei used for these experiments. **(B)** Sperm nuclei were incubated in egg extract, at 10,000/μl, in the presence and absence of 40 ng/μl double-strand break containing DNA. The chromatin was isolated at 0, 15, 30, and 90 minutes and probed for the presence of ATM phosphorylated on the equivalent of Ser1981. The illustrated lanes in (B) were from the same experiment and gel, but spliced together for clarity. These data establish that manipulation of the egg extract and sperm nuclei during preparation does not activate ATM. In contrast, ATM activation, as seen by autophosphorylation of the residue equivalent to Ser1981 was rapidly induced by addition of DSB-containing DNA. This is consistent with the absence of Histone H2AX phosphorylation in chromatin following incubation in extracts[2].

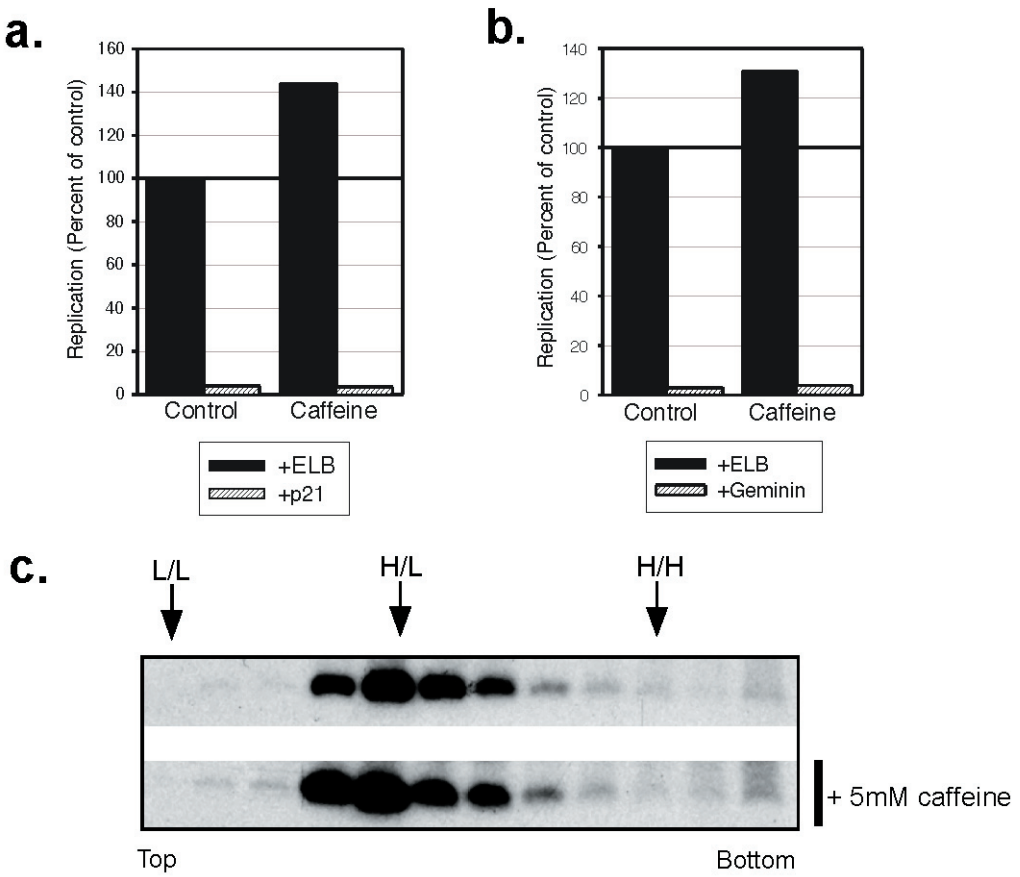

Figure S2 Caffeine does not overcome the block to origin firing by $p21^{cip1}$ and Geminin or induce re-replication. **(A)** Nuclei were incubated in extract with ^{32}P-dATP at 2,000/μl for 45 minutes in the presence of ELB (solid) or of 50 ng/μl $p21^{cip1}$ protein (hatched) and in the presence or absence of 5 mM caffeine. Incorporation was quantitated and plotted as a percent of the ELB control. **(B)** Nuclei were incubated in extract with ^{32}P-dATP at 2,000/μl for 45 minutes in the presence of ELB (solid) or of 50 ng/μl Geminin protein (hatched) and in the presence or absence of 5 mM caffeine. Incorporation was quantitated and plotted as a percent of the ELB control. Caffeine did not promote incorporation in the presence of $p21^{cip1}$ protein or in the presence of Geminin, demonstrating that the effect of caffeine was specific to origin-dependent and cell-cycle regulated DNA replication. **(C)** Nuclei were incubated in interphase extract with 0.75 mM bromodeoxyuridine triphosphate and ^{32}P-dATP at 2,000/μl for 2 hours. The reaction was stopped and deproteinized. The replicated products were then run through a 1.72 g/cm^3 CsCl gradient, fractions collected from the top of the tube, ethanol precipitated, and run on a 0.8% agarose gel. H/L (heavy/light) represents the peak of BrdU hemi-substituted DNA, showing only one round of semi-conservative DNA replication. L/L (light/light) and H/H (heavy/heavy) approximately represent the peaks of un-substituted and fully-substituted DNA, respectively. We did not observe re-replication after treatment of extract with caffeine, although the heavy/light peak from the CsCl gradient, indicating semi-conservative DNA replication, was higher in the presence of caffeine, consistent with increased replicon density.

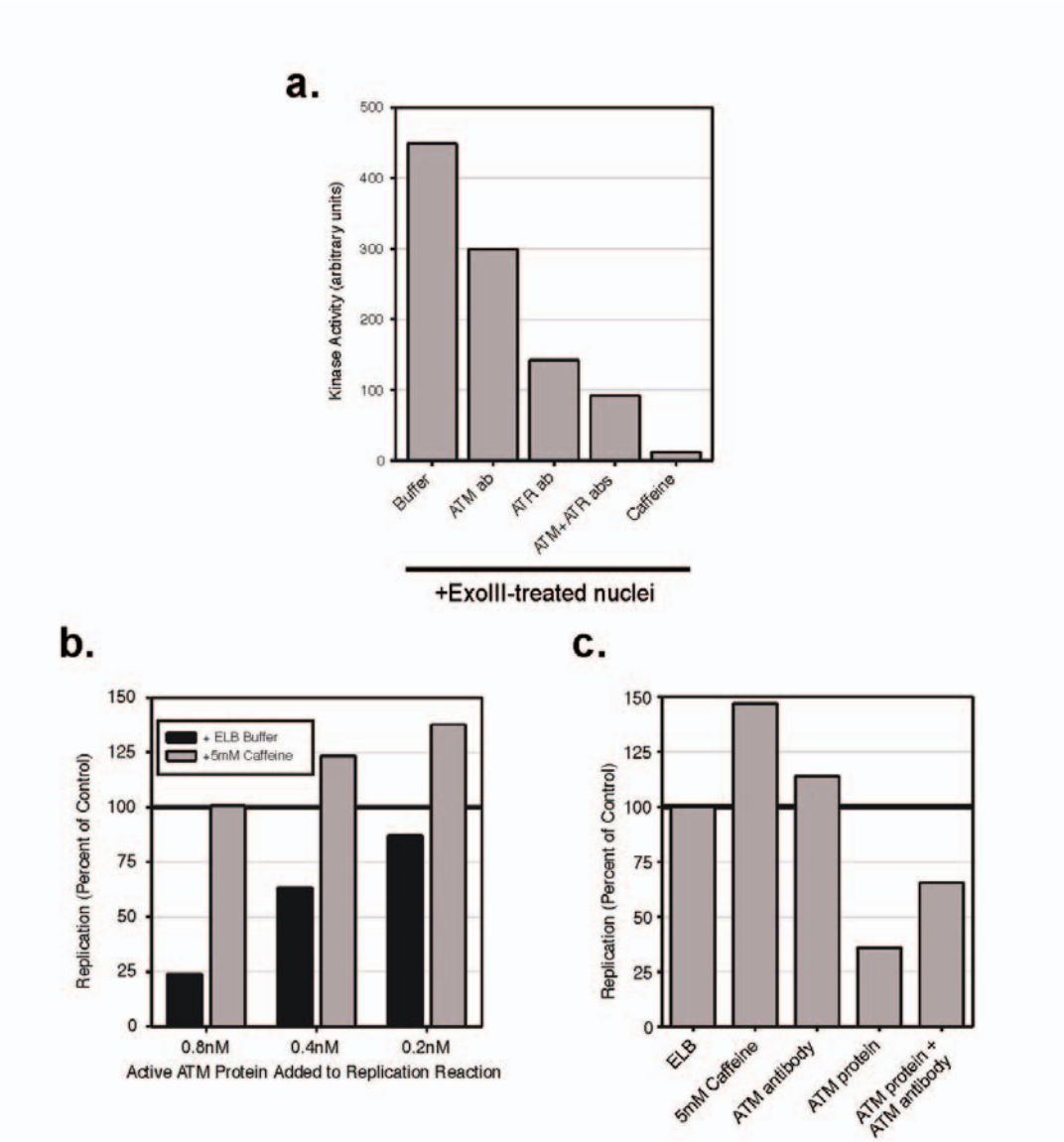

Figure S3 ATM- and ATR-neutralizing antibodies specifically inhibit kinase activity after double-strand breaks and single-strand gaps, respectively, and ATM protein rescues the effect of caffeine and ATM-neutralizing antibody. **(A)** Interphase egg extract was incubated with 50 ng/μl double-strand break containing DNA for 30 minutes, in the absence or presence of 20% v/v ATM-neutralizing antibody, ATR-neutralizing antibody, both antibodies, or 5mM caffeine, as indicated, and assayed for kinase activity directed towards a peptide derived from the C-terminal tail of Histone H2AX. This shows that DSBs triggered phosphorylation of the H2AX peptide. This phosphorylation was most sensitive to ATM inhibition, but was also sensitive to ATR inhibition[2], consistent with ATM, and to a lesser extent, ATR, activation following DSB addition. **(B)** Interphase egg extract was incubated with Exonuclease-III treated sperm nuclei at 10,000/μl for 30 minutes, in the absence or presence of 20% v/v ATM-neutralizing antibody, ATR-neutralizing antibody, both antibodies, or 5mM caffeine, as indicated, and assayed for kinase activity directed towards a peptide derived from Histone H2AX. This shows that ATR-neutralizing antibodies dramatically inhibited the H2AX-peptide phosphorylation in extract containing Exonuclease-III treated chromatin, while ATM-neutralizing antibodies had a modest effect, and caffeine completely inhibited the activity. These data confirm the specificity of the ATM- and ATR-neutralizing antibodies that we had previously established[1,3]. **(C)** Nuclei were incubated in extract with ^{32}P-dATP at 10,000/μl for 60 minutes with either ELB buffer (black bars) or 5 mM caffeine (gray bars) and active ATM protein at 0.8 nM, 0.4 nM, or 0.2 nM final concentrations. Replication products were quantitated and plotted as percent of the buffer control. **(D)** Nuclei were incubated in extract with ^{32}P-dATP at 10,000/μl for 60 minutes with ELB buffer, 5 mM caffeine, 20% v/v ATM-neutralizing antibody[4], 0.8 nM active ATM protein, or 0.8 nM active ATM protein and 20% v/v ATM-neutralizing antibody. Replication products were quantitated and plotted as percent of the buffer control. Panels C and D are from the same extract and the same control was used for quantitation. These data establish that purified, active, human ATM protein[5] rescued the effect of caffeine on DNA replication in a dose-dependent manner and also rescue the stimulation of DNA replication triggered by neutralizing ATM antibodies.

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